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Quantitative analysis and process monitoring of site-specific glycosylation microheterogeneity in recombinant human interferon-γ from Chinese hamster ovary cell culture by hydrophilic interaction chromatography

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Abstract

A chromatographic method was developed for quantitative analysis of site-specific microheterogeneity of the two *N*-linked glycosylation sites in recombinant human interferon-γ produced from Chinese hamster ovary (CHO) cell culture. After the interferon-γ was harvested by affinity chromatography, the tryptic digestion was carried out. The two glycopeptide pools, isolated from reversed-phase chromatography of tryptic digestion of interferon-γ, were subjected to further separation by hydrophilic interaction chromatography. Each peak in the chromatograms was identified by matrix-assisted laser desorption ionization and time-of-flight mass spectrometry (MALDI–TOF–MS). The overall elution order of the glycopeptides was the following: neutral glycopeptides, monosialylated glycopeptides, bisialylated glycopeptides, trisialylated glycopeptide and tetrasialylated glycopeptides. Based on the integrated peak area for each compound in the chromatograms, the percentage for each glycan was utilized to quantify the glycosylation pattern of the interferon-γ. Finally, sialylation and antennarity structure percentages at the two glycosylation sites were chosen as the quality indicators in process monitoring of interferon-γ production from a serum-free suspension-batch CHO culture.

Keywords: Chinese hamster ovary cell culture; Interferon; Glycopeptides

1. Introduction

The majority of recombinant therapeutic proteins are glycoproteins produced from mammalian cell cultures. The oligosaccharide residues on the glycoproteins could exert significant effects on many properties of the glycoproteins. These include antigenicity, solubility, pharmacokinetics, biological activity and protease resistance [1,2]. It is essential to characterize the glycosylation pattern for safety, efficacy and consistency of the glycoproteins. Recently the significance of monitoring the glycosylation pattern during cell cultivation is well recognized [3–5]. It has been reported that the environment in which cells are cultured could affect the glycosylation pattern [6,7]. This type of data from process monitoring provides an explanation on the dynamics of glycosylation as well as providing a possible way of manipulating glycosylation pattern.

Characterization of the glycosylation pattern is a

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challenging task because there is a population of glycans with different carbohydrate structures at each potential glycosylation site (i.e. glycoform microheterogeneity). Furthermore, it is necessary to obtain site-specific information because each potential glycosylation site could encounter a different steric environment [8,9]. The best strategy for characterization of site-specific glycosylation microheterogeneity is by generating the glycopeptides from enzymatic digestion of glycoproteins and then analyzing the glycopeptides by a variety of techniques [10–13], i.e., matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI–TOF–MS) and electrospray mass spectrometry (ES–MS). MALDI–TOF–MS is popular because of its speed, high sensitivity and ability to analyze the peptide mixtures. But when analyzing glycopeptides with negatively charged glycans, i.e. sialylated glycopeptides, it is difficult to acquire quantitative information because a negatively charged oligosaccharide would influence the ionization efficiency of the glycopeptide [14] and source fragmentation would occur due to the labile glycosidic linkage of sialic acid [15]. In this paper, a hydrophilic-interaction chromatography (HILIC) method [16] was developed to analyze the glycopeptides from recombinant interferon-γ. Process monitoring of site-specific glycosylation microheterogeneity was then carried out for the recombinant interferon-γ produced during a batch culture of Chinese hamster ovary (CHO) cells.

2. Experimental

2.1. CHO cell culture

Recombinant human IFN-γ was produced by a CHO cell line cotransfected with genes for dihydrofolate reductase and human IFN-γ and selected for growth in the presence of 2.5×10^{-7} *M* methotrexate (Sigma, St. Louis, MO, USA) [4,5]. After the cell line was adapted into serum-free SFM-CHO-II medium (Gibco, Gaithersburg, MD, USA) supplemented with 2.5×10^{-7} *M* methotrexate, 10 units ml^{-1} penicillin (Sigma), and 10 $\mu g\,ml^{-1}$ streptomycin (Sigma), a 100-ml suspension-batch culture was initiated in a spinner flask (50 rpm) in a 37°C incubator (10% carbon dioxide). The inoculating density of the CHO cell culture was 4.2×10^{5} ml^{-1} and the maximum viable cell density of 2.5×10^{6} ml^{-1} was obtained in 96 h. Cell density and viability were measured on a Neubauer hemacytometer (Reichert, Buffalo, NY, USA) with trypan-blue dye exclusion. The cell culture lasted 168 h and at every 24 h, the supernatant was collected for analysis.

2.2. Chromatography

All chromatographic separations were conducted on an INTEGRAL Micro-Analytical Workstation (PerSeptive Biosystems, Framingham, MA, USA). The supernatant was first filtered (0.22 μm, Sterile Acrodisc, Gelman Sciences, Ann Arbor, MI, USA) and then loaded onto a 150×0.76 mm I.D. column, packed with immunoaffinity resin immobilized with monoclonal antibody against the recombinant human interferon-γ (Reselute-γ, Celltech, Slough, UK) previously equilibrated with a loading buffer of pH 7.2, 10 m*M* sodium phosphate (Mallinckrodt, Paris, KY, USA) and 150 m*M* NaCl (Mallinckrodt) at 0.2 $ml\,min^{-1}$. The bound IFN-γ was eluted by a step gradient of 12 m*M* HCl and 150 m*M* NaCl at 0.2 $ml\,min^{-1}$. The IFN-γ fraction was monitored at 220 nm, manually collected and immediately mixed with an equal volume of trypsin digestion buffer of 250 m*M* Tris (Sigma), 500 m*M* urea (Sigma), pH 8.5. Based on the integrated peak area, the concentration of IFN-γ was determined by the method developed by Harmon et al. [4].

After IFN-γ was digested by trypsin (1:5 ratio, enzyme mass:substrate mass) (sequencing grade, Boehringer Mannheim, Indianapolis, IN, USA) at 37°C for 24 h, reversed-phase separation of the tryptic peptides was performed on a 250×1 mm I.D. Vydac C_{18} analytical HPLC column (The Separation Group, Hesperia, CA, USA). The digestion solution was loaded onto the column at 50 $\mu l\,min^{-1}$, and washed with 95% HPLC-grade water (EM Science, Gibbstown, NJ, USA) and 5% acetonitrile (EM Science) containing 0.1% trifluoroacetic acid (TFA; Pierce, Rockford, IL, USA) and eluted by a 30-min gradient to 5% HPLC-grade water and 95% acetonitrile with 0.085% TFA at a flow-rate of 50 $\mu l\,min^{-1}$. The glycopeptide fractions were monitored at 220

nm, collected and then identified by MALDI–TOF–MS.

In the hydrophilic interaction chromatography (HILIC) separation, the glycopeptide fractions from RPC were mixed with the mobile phase A of 85% acetonitrile, 15% water, 10 m*M* triethylamine (Fisher Scientific, Fair Lawn, NJ, USA) pH 6.0 and then the resulting solution was loaded onto a 150×1 mm I.D. polyhydroxyethyl Aspartamide column (PolyLC, Columbia, MD, USA) at 0.1 ml min^{-1} with the mobile phase A. Monitored at 220 nm, the glycopeptides were eluted at 50 μl min^{-1} with a 60-min gradient from the mobile phase 100% A to the mobile phase 100% B of 20% acetonitrile, 80% water, 10 m*M* triethylamine, 25 m*M* sodium perchlorate (EM Science) and pH 6.0. The fractions were manually collected and then identified by MALDI–TOF–MS.

2.3. MALDI–TOF mass spectrometry

MALDI–TOF mass spectrometry was performed on a Voyager BioSpectrometry Workstation (PerSeptive Biosystem). A 1-μl sample from RPC or HILIC separation was mixed with 1 μl of the matrix solution of 7 mg ml^{-1} of 2,5-dihydroxybenzoic acid (Aldrich, Milwaukee, WI, USA) in 50% water, 50% acetonitrile, 0.1% TFA. One microliter of the mixture was deposited into a well on the MALDI sample plate and air-dried at room temperature before being introduced into the mass spectrometer. Positive-ion linear TOF detection was performed with an accelerating voltage of 28 125 V. Each spectrum was obtained by averaging the data of 35–723-ns pulses of the 337-nm nitrogen laser and smoothed by a 19-point Savitzky–Golay filter. The external calibration was conducted with a mixture of bradykinin (M_r 1060.2; Sigma) and oxidized insulin B chain (M_r 3495.9; Sigma).

3. Results and discussion

Recombinant human IFN-γ produced from CHO cells has two potential *N*-linked glycosylation sites at Asn^{25} and Asn^{97}. The individual glycan structures located at each glycosylation site have already been identified by 2-D ^1H NMR [17], MALDI–TOF–MS [18]. Characterization of site-specific glycosylation microheterogeneity was achieved by carrying out analysis on the proteolytic glycopeptides from glycoproteins. As shown in Fig. 1, the viable cell

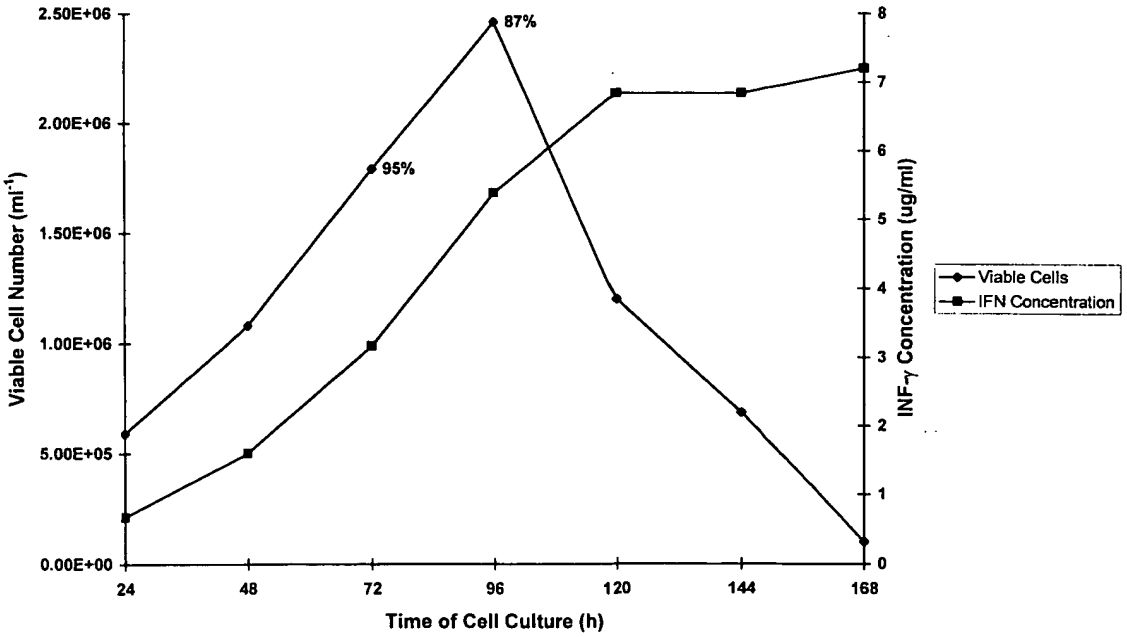


Fig. 1. Viable cell density and IFN-γ concentration during a suspension-batch CHO cell culture.

density showed exponential growth, reached the maximum after 96 h of cultivation and then declined rapidly; IFN-γ concentration increased from 0.67 $\mu g\,ml^{-1}$ after 24 h of fermentation to its maximum 6.9 $\mu g\,ml^{-1}$ after 120 h and remained relatively constant afterwards. The IFN-γ following 120 h of suspension CHO batch culture was purified from the cell culture supernatant by affinity chromatography and then digested by trypsin. It has been shown that after tryptic digestion, the two potential glycosylation sites would be separated into two individual peptide fragments [4,18].

The HILIC chromatograms for the glycopeptides of Asn^{97} and Asn^{25} are shown in Figs. 2 and 3, respectively, and the molecular mass of each peak in the two chromatograms was determined by MALDI–TOF–MS. The MALDI–TOF–MS spectra for peak 1 and 7 from the HILIC chromatogram for the glycopeptides of Asn^{97} and those for peak 3 and 7 from the HILIC chromatogram for the glycopeptides of Asn^{25} are shown in Fig. 4A–D, respectively. The glycan structures of these glycopeptides could be identified by comparing the mass difference between the observed mass of a glycopeptide from MALDI–TOF–MS and the known mass of its peptide fragment (i.e. 1522.7 and 2252.5 for the peptides of Asn^{97} and Asn^{25} [4,18]) with the expected mass shift due to the glycans commonly observed in the glycoproteins produced from CHO cell culture. The glycan structures for the glycopeptides of Asn^{97} and Asn^{25} are listed in Tables 1 and 2. Based on the integrated peak areas for the identified peaks in both chromatograms, the percentages for each glycan are also presented in these tables. The most abundant glycans are biantennary with full sialylation, i.e. 45.2% and 39.8%, respectively, for the glycans of Asn^{25} and Asn^{97}. The triantennary glycans with full sialylation accounted for 20.5% in the glycans of Asn^{25} and the biantennary glycans with one sialic acid for 33.8% in the glycans of Asn^{97}. Another distinct observation is that all the glycans of Asn^{25} are fucosylated while those from Asn^{97} are not.

From the two HILIC chromatograms, it can be concluded that the glycopeptide with a higher sialic acid content has longer retention time. For example, in the chromatogram of glycopeptides of Asn^{25}, the glycopeptides are eluted in the sequence of monosialo, bisialo, trisialo and tetrasialo glycans with

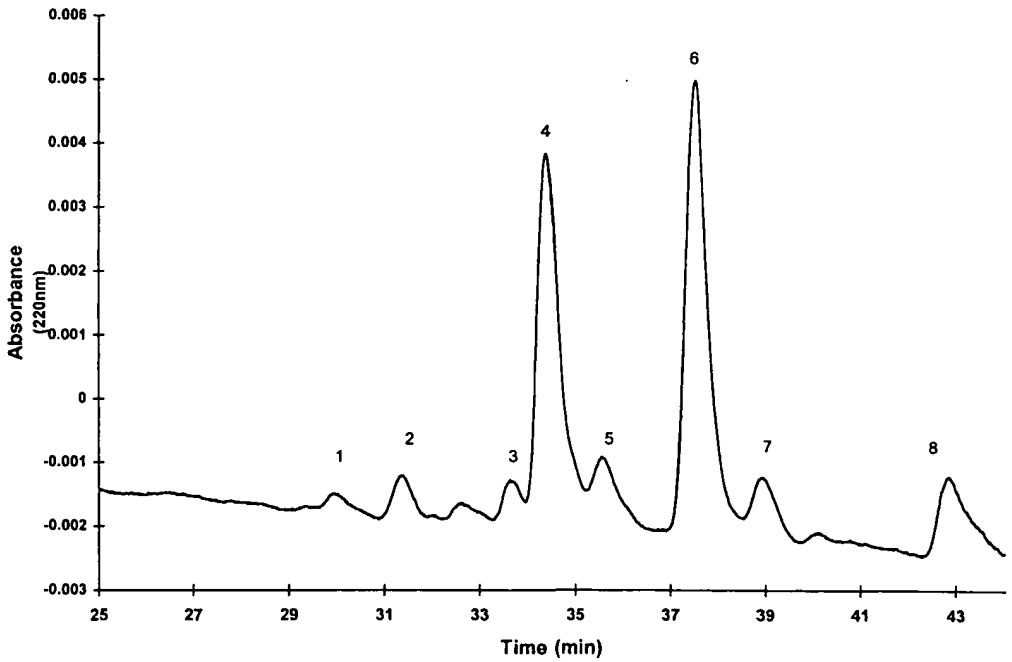


Fig. 2. HILIC chromatogram for the tryptic glycopeptides of Asn^{97} from recombinant human interferon-γ following 120 h of a CHO cell batch culture.

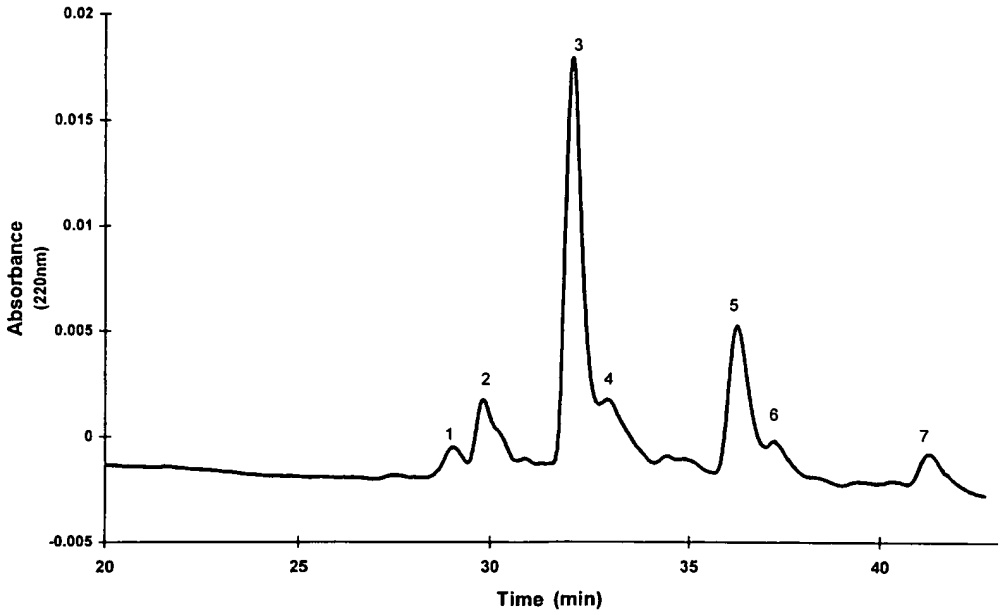


Fig. 3. HILIC chromatogram for the tryptic glycopeptides of Asn^{25} from recombinant human interferon-γ following 120 h of a CHO cell batch culture.

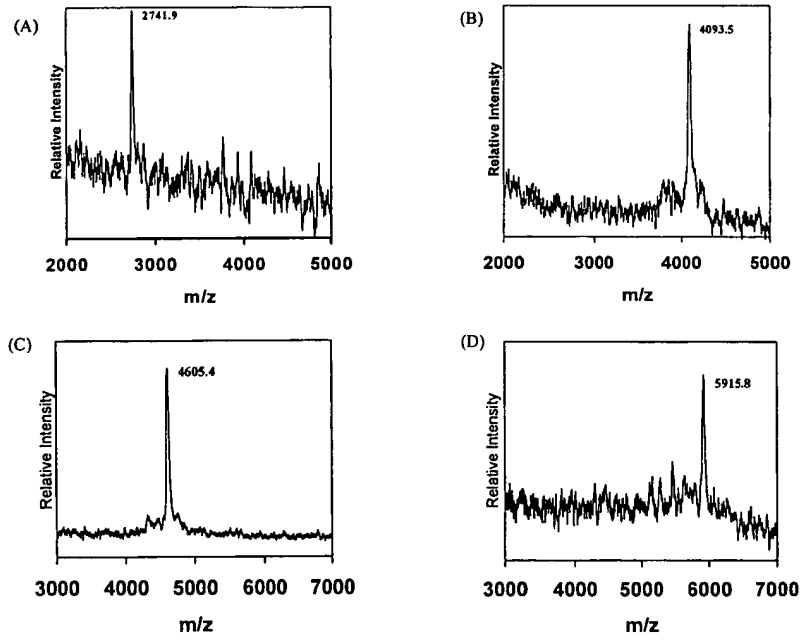


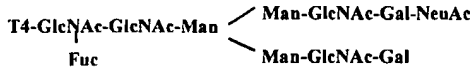
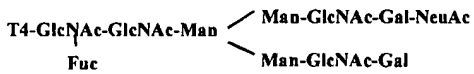
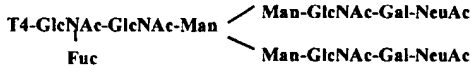
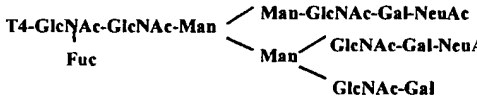
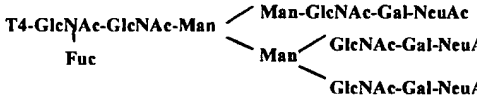
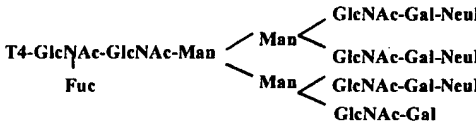
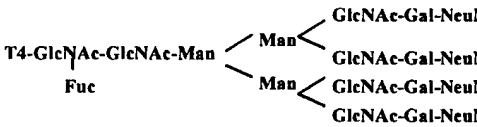
Fig. 4. MALDI–TOF–MS mass spectra (A) for peak 1 and (B) for peak 7, and (C) for peak 3 and (D) for peak 7 from HILIC separation of the tryptic glycopeptides of Asn^{97} and Asn^{25}, respectively, from recombinant human interferon-γ following 120 h of a CHO cell batch culture.

Table 1
Oligosaccharide structures for Asn^{97} in IFN-γ identified by MALDI–TOF–MS. The numbers indicate the labelled peaks shown in Fig. 2.

Peak #	Glycopeptide Structure	$(M+H)^+$ Expected Mass	$(M+H)^+$ Observed Mass	Peak Area%
1	T97-GlcNAc-GlcNAc-Man(Man)(Man(Man)(Man))	2740.6	2741.9	2.3
2	T97-GlcNAc-GlcNAc-Man(Man-GlcNAc-Gal)(Man-GlcNAc-Gal)	3147.0	3148.2	3.5
3	T97-GlcNAc-GlcNAc-Man(Man-GlcNAc-Gal-NeuAc)(Man-GlcNAc-Gal)	3438.3	3440.2	3.1
4	T97-GlcNAc-GlcNAc-Man(Man-GlcNAc-Gal-NeuAc)(Man-GlcNAc-Gal)	3438.3	3439.3	30.7
5	T97-GlcNAc-GlcNAc-Man(Man-GlcNAc-Gal-NeuAc)(Man(GlcNAc-Gal)(GlcNAc-Gal))	3803.6	3805.3	7.2
6	T97-GlcNAc-GlcNAc-Man(Man-GlcNAc-Gal-NeuAc)(Man-GlcNAc-Gal-NeuAc)	3729.6	3726.2	39.8
7	T97-GlcNAc-GlcNAc-Man(Man-GlcNAc-Gal-NeuAc)(Man(GlcNAc-Gal-NeuAc)(GlcNAc-Gal))	4094.9	4093.5	5.6
8	T97-GlcNAc-GlcNAc-Man(Man-GlcNAc-Gal-NeuAc)(Man(GlcNAc-Gal-NeuAc)(GlcNAc-Gal-NeuAc))	4386.2	4388.0	7.9

respect to the carbohydrate structure of the glycopeptide. The selectivity of the HILIC separation is dominated by the number of sialic acids on the glycopeptides, which indicates that the initial high concentration of acetonitrile would promote the hydrophilic interaction between the stationary phase and the "contact region" [16] of the glycan on the glycopeptide. The "contact region" concept could be utilized to explain the selectivity difference observed in HILIC and RPC separation. The contact region in the RPC separation is the peptide portion of the glycopeptides and this region is the oligosaccharide part in the HILIC separation. Therefore RPC could not readily resolve the glycopeptides because of their identical amino acid sequence. Furthermore, HILIC could separate different glycans with the same number of sialic acids. As shown in Fig. 2, the bisialo biantennary glycopeptide (peak 6) is resolved from bisialo triantennary glycopeptide (peak 7), although baseline resolution was not achieved. Another interesting observation is that there are two peaks with the same masses in the HILIC separation, i.e., peak 3 and peak 4 in Fig. 2. This suggests the separation of two monosialo biantennary structures based on the branch of sialylation, i.e., sialic acid on either MAN(α1→3) or MAN(α1→6) arm, often

Table 2
Oligosaccharide structures for Asn[25] in IFN-γ identified by MALDI–TOF–MS. The numbers indicate the labelled peaks shown in Fig. 3.

Peak #	Glycopeptide Structure	(M+H)+ Expected Mass	(M+H)+ Observed Mass	Peak Area%
1		4314.2	4315.3	3.2
2		4314.2	4315.5	9.8
3		4605.5	4605.4	45.2
4		4970.8	4968.9	9.9
5		5262.1	5266.1	20.5
6		5627.4	5628.3	6.1
7		5918.7	5915.8	5.3

observed in glycoproteins secreted from CHO cells [5]. It was hypothesised that in HILIC chromatography, there is a partition mechanism between the hydrophobic mobile phase and a layer of mobile phase enriched with water and immobilized on the stationary phase [16]. The location of sialic acid on either of the two arms would offer different contact orientation for the glycans to interact with the stationary phase, which results in slightly different hydrophilicities. Alpert et al. [19] provided a detailed explanation based on the observation of separation of carbohydrates differing in the position of linkage in HILIC chromatography.

Sialylation has a significant effect on the pharmacokinetics of therapeutic glycoproteins [20], besides other properties: solubility [21], thermal stability [22], protease resistance [23], antigenicity [24]. Sialylation could increase the circulatory lifetime of glycoproteins by shielding them from being recognized at the exposed galactose or *N*-acetylgalactosamine residues by the asialoglycoprotein receptors on the surface of the blood hepatocytes and degraded by the hepatocytes in the lysosomes [20]. Asialoglycoprotein antennarity of the glycans would affect the clearance rate of the glycoproteins. Since a precise three-point binding between the oligosaccharide and the hepatic receptor is prerequisite [25,26], the asialoglycoproteins with trianten-

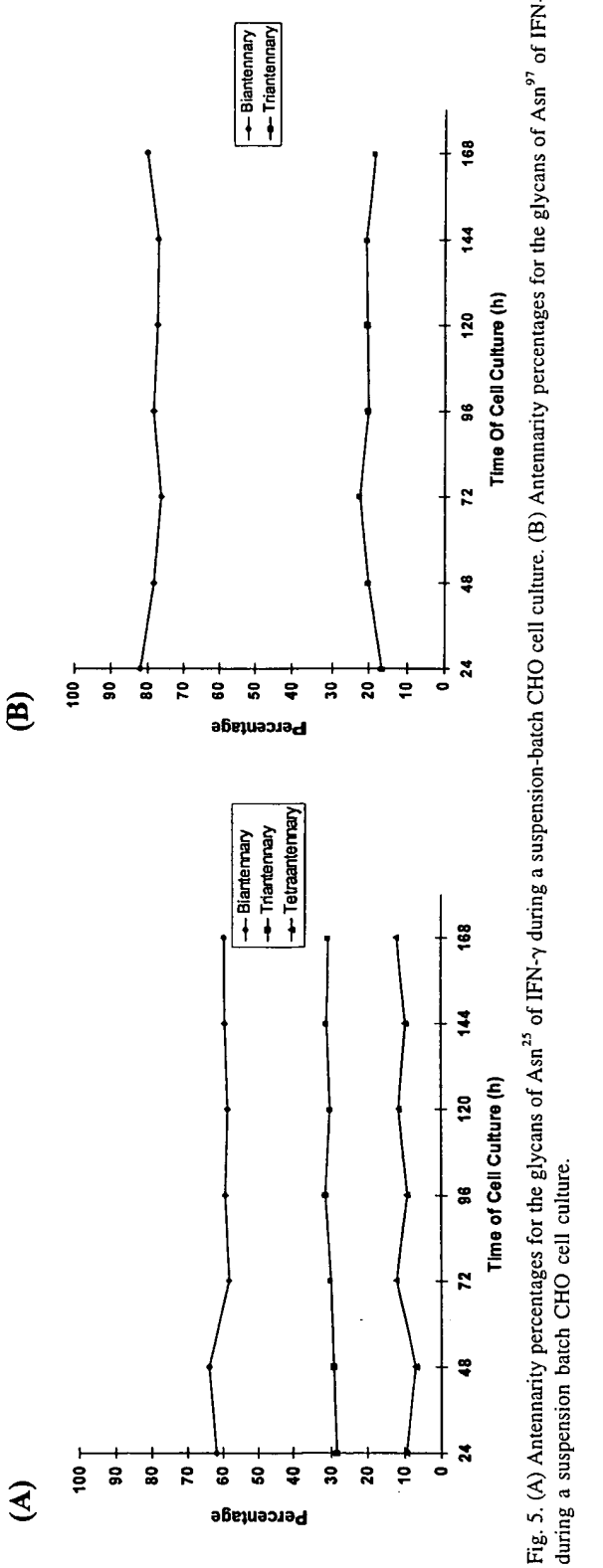


Fig. 5. (A) Antennarity percentages for the glycans of Asn^{25} of IFN-γ during a suspension-batch CHO cell culture. (B) Antennarity percentages for the glycans of Asn^{97} of IFN-γ during a suspension batch CHO cell culture.

nary and tetriantennary glycans have much faster clearance rate than those with biantennary glycans [24,25]. Because of the significance of sialylation and antennarity, they are chosen as the indicators of the IFN quality. The sialylation percentages, calculated based on the definition by Gu et al. [5], are 88.8% and 73.5%, respectively, for the IFN-γ at Asn^{25} and Asn^{97} following 120 h of cell culture. The percentages for each antennarity were calculated to be the following: biantennary, triantennary, tetraantennary glycans accounted for 58.2, 30.4 and 11.4%, respectively, of the glycans of Asn^{25}, and the two former glycans for 77.0 and 20.7%, respectively, of the glycans of Asn^{97}. All the above observations agree with previously published results regarding the glycosylation of CHO-derived IFN-γ, i.e., higher average antennarity for the glycans of Asn^{25} [4,18], higher sialylation percentage for the glycans of Asn^{25} [5] and fucosylation of the glycans of Asn^{25} [4,18]. Since both Asn^{25} and Asn^{97} should have a similar supply of nucleotide sugar precursors, the glycosylation differences would be attributed to the steric local environment the two sites experienced. It has been suggested that the Asn^{25} site is located in the middle of the random coil while Asn^{97} is in a restricted helix [27,28], which makes Asn^{25} more accessible for the *N*-acetylglucosaminyltransferase IV sialyltransferase and fucosyltransferase. Although not significant, approximately 2% of the glycans of Asn^{97} has a high mannose content. This may indicate that restricted oligosaccharide processing by *N*-acetylglucosaminyltransferase and Golgi α-mannosidase II occur in the medial-Golgi for Asn^{97} due to the steric hindrance.

For the above analysis only 6 μg of interferon-γ was consumed. Therefore, sialylation and antennarity percentages for both Asn^{25} and Asn^{97} could be monitored throughout the cultivation process. As shown in Fig. 5 (A) and (B), the relative percentages of biantennary, triantennary and tetraantennary structures of Asn^{97} and Asn^{25} obtained from the HILIC chromatograms, did not change significantly for either site during the course of the cultivation, which means that there are no substantial obstacles in the glycosylation pathway. As shown in Fig. 6, sialylation percentages for both Asn^{25} and Asn^{97} remain relatively constant for the first 72 h and were gradually decreased from 92.4% and 78.3% to 84.9% and 71.6% after 168 h, respectively, for Asn^{25} and Asn^{97}. The loss of sialylation was coincidental with the onset of cell death following 96 h of fermentation, i.e., 87% cell viability, which substantiates the finding by Gu et al. [5] that the incomplete sialylation was due to the incomplete intracellular sialyla-

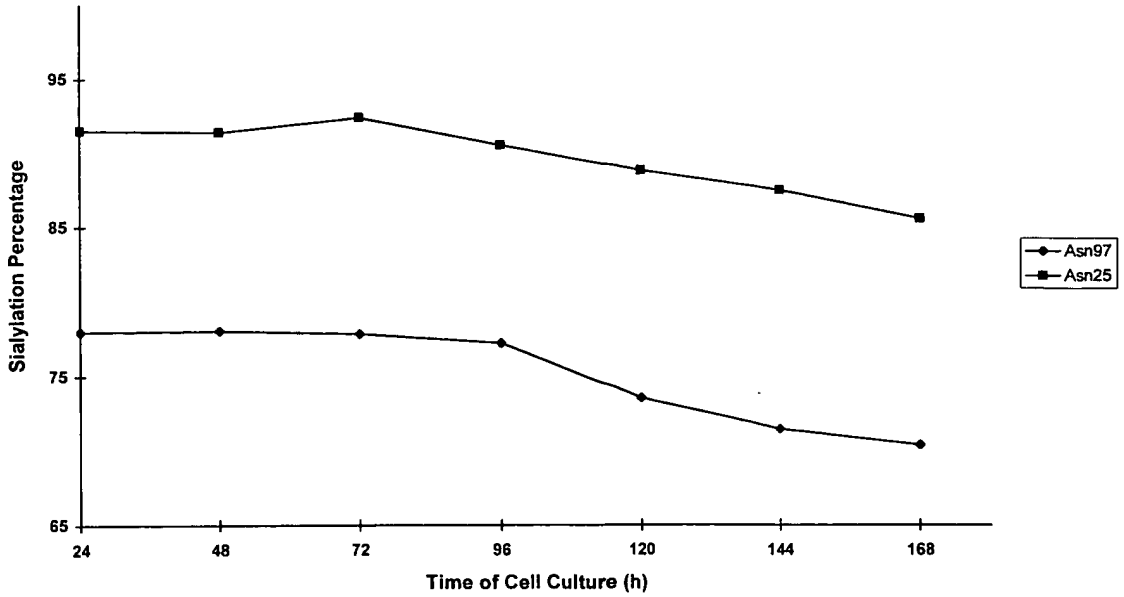


Fig. 6. Sialylation percentages for the glycans of Asn^{97} and Asn^{25} of IFN-γ during a suspension batch CHO cell culture.

tion initially and then the later desialylation by sialidase released by the lysed cells.

4. Conclusion

A hydrophilic interaction chromatography method has been developed for monitoring the site-specific glycosylation microheterogeneity of recombinant human IFN-γ produced from a suspension-batch CHO culture. Based on the integrated peak area, the relative percentages for the individual glycans can be obtained. Because sialylation and antennarity are the two common glycosylation microheterogeneities for glycoproteins secreted from CHO cells, the sialylation percentages and antennarity percentages for both glycosylation are monitored throughout the cell culture. The antennarity percentages of both Asn^{25} and Asn^{97} stay relatively constant while the sialylation percentages at both sites begin to drop as soon as the cells start dying. To maintain the consistency of the IFN-γ product, IFN-γ should be harvested as soon as the cells begin to die or sialidase inhibitors could be added to minimize the activity of sialidase.

Acknowledgements

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